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(54) Vascular specific regulatory elements contained in the desmin 5' flanking region

(57) The present invention concerns a DNA sequence isolated from the 5' flanking region of a desmin gene located between nucleotides -10000 to -1000 relative to the transcription initiation site, which contains regulatory elements specific to arterial smooth muscle cells. It also provides an expression cassette using such a DNA sequence to control expression of a gene of interest. Finally, the invention relates to a recombinant vector, a viral particle, an eukaryotic host cell, a pharmaceutical composition comprising said expression cassette and their therapeutic or prophylactic use as well as a transgenic animal having incorporated in its genome said expression cassette.

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Description

[0001] The present invention concerns a DNA sequence isolated from the 5' flanking region of a desmin gene located between nucleotides -10000 to -1000 relative to the transcription initiation site, which contains regulatory elements specific to arterial smooth muscle cells. It also provides an expression cassette using such a DNA sequence to control expression of a gene of interest. Finally, the invention relates to a recombinant vector, a viral particle, an eukaryotic host cell, a pharmaceutical composition comprising said expression cassette and their therapeutic or prophylactic use as well as a transgenic animal having incorporated in its genome said expression cassette. The present invention relates to the field of tissue-specific gene expression and has numerous applications particularly in human diseases. It is of very special interest in relation to prospect for gene therapy, especially in the cardiovascular field.

[0002] Gene therapy can be defined as the transfer of genetic material into a cell or an organism to treat or prevent a genetic or acquired disease. The possibility of treating human diseases by gene therapy has changed in a few years from the stage of theoretical considerations to that of clinical applications. The first protocol applied to man was initiated in the USA in 1990 on a patient who was genetically immunodeficient as a result of a mutation affecting the gene encoding adenine deaminase (ADA). The relative success of this first experiment encouraged the development of this technology for various genetic and acquired diseases. The large majority of the current protocols employ vectors to carry the therapeutic gene to the cells to be treated. Numerous viral or synthetic vectors have been developed during these last years. Generally speaking, their structure, organization and biology are described in the literature available to a person skilled in the art.

[0003] However, under certain circumstances, the broad host range of these vectors can represent a major limitation for their use. Their lack of specificity could lead to a widespread expression of therapeutic genes which might be harmful to the patient especially when cytotoxic genes are transferred. On the other hand, the efficiency of the treatment could also be reduced by the overall dilution of the vector in the organism. Several strategies have been proposed to restrict expression to the category of cells to be treated. The use of tissue-specific regulatory elements represent an attractive approach to overcome this problem.

[0004] Cardiovascular diseases represent a major target for gene therapy approaches since they are a leading cause of mortality in most of the industrialized countries. The general concept is based on the delivery of therapeutic genes locally or in the systemic circulation. A promoter capable to target expression in vascular smooth muscle cells might be beneficial to avoid the potential side effects inherent to a widespread gene expression. The existence of muscle cell specific promoters has been described in the literature. However, expression is rarely restricted to a particular muscle type. Thus, there is still a need for specific regulatory elements that are functional *in vivo* and are specific of subtypes of smooth muscle cells to allow treatment of cardiovascular pathologies.

[0005] The transcriptional regulation of tissue-specific genes is usually dependant of the interaction between *trans*-acting transcription factors and *cis*-acting sequences present in the promoter, some of them being tissue-specific. A first family of myogenic *trans*-acting factors (MyoD) responsible for skeletal muscle expression was characterized at the end of the eighties (MyoD, myogenin, myf-5, MRF4...). Their binding to a *cis*-acting element called E box (consensus CANNTG) promotes transcriptional activation of sequences controlled by promoters including such a regulatory element. In addition, other transcription factors have also been involved in activation of muscle-specific genes, such as CArG box binding factor (or SRF), M-CAT binding protein, and MEF-2 factors....

[0006] The desmin gene encodes a cytoskeletal protein constitutive of intermediate filaments which occur in the cytoplasm of most muscular cells. Desmin expression is initiated at a very early stage of embryogenesis in the myogenic lineage and maintained in adult stage in all muscular type cells (cardiac, skeletal and smooth muscle cells). The desmin gene and its promoter region were cloned and sequenced for several species (Li et al., 1989, Gene 78, 243-254 reporting the human gene sequence. Quax et al., 1984, Proc Natl Acad Sci USA 81, 5970-5974 et Quax et al., 1985, Cell 43, 327-338 for the hamster desmin gene; Li et al., 1996, Dev Biol. 175, 362-366 for the mouse gene).

[0007] Previous studies have revealed a complex regulatory mechanisms to promote the temporal and tissue-specific expression of the desmin gene during myogenesis. Positive and negative control regions were identified in the first 1000 bp of the 5' flanking region of the human desmin gene. In particular, a muscle-specific enhancer was localised between nucleotides (nt) -973 to -693 (relative to the transcriptional initiation site or cap site representing +1). Its capability to activate either homologous (desmin) or heterologous promoters in a manner independant of orientation, position or distance was demonstrated by *in vitro* transfection assays using a series of CAT expression vectors containing different lengths of 5' upstream desmin region (Li and Paulin, 1991, J. Biol. Chem. 266, 6562-6570). Transgenic mice analysis showed that 1 kb of the desmin 5' upstream sequence are sufficient to direct muscle-specific and developmentally regulated expression in skeletal muscles and suggested a distinct regulation for cardiac and smooth muscle expression (Li et al., 1993, Development 117, 947-959).

[0008] Sequence analysis of the mouse desmin promoter region also demonstrated the presence of several potential *cis*-acting elements. Three MyoD binding sites, one MEF-2 binding site, one M-CAT motif and several GC boxes as well as a muscle-specific enhancer were characterized within the 976pb preceeding the transcription initiation site (Li

and Capetanaki, 1993, Nucleic Acids Res. 21, 335-343). The proximal E box and enhancer MEF-2 binding sites as well as their location relative to the TATA box are crucial for high levels expression (Li and Capetanaki, 1994, EMBO J. 13, 3580-3589). Concerning the hamster desmin gene, Van de Klundert et al (1994, J. Biol. Chem. 269, 220-225) have reported the importance of the consensus E box element located in the direct vicinity of the cap site (-80) for promoter activity whereas the contribution of a second E box situated at -825 is only moderate.

[0009] Documents of the prior art have focused on the proximal portion of the desmin promoter and disclose its capability to induce expression of heterologous sequences mainly in skeletal muscles. The present invention goes beyond this teaching in providing regulatory elements specific for expression in arterial smooth cells present within a more distal part of the desmin promoter. Recombinant constructions driven by a promoter comprising the region -4006 to -2495 of the mouse desmin gene show a specificity of expression restricted to arterial smooth muscle cells. Sequence analysis reveals the presence of several potential transcription factors binding sites including 5 CArG-like sequences known to regulate many muscle specific genes especially cardiac and skeletal α -actin gene. Furthermore sequence comparison between human and murine desmin 5' flanking sequences shows 4 conserved regions between the two species, one being located in the arterial smooth muscle specific putative region. This region has special interest in cardiovascular diseases field.

[0010] Accordingly a first object of the present invention is an isolated DNA sequence comprising all or pan of the 5' flanking region of a desmin gene located between approximately nucleotides -10000 to -1000 relative to the transcription initiation site of said desmin gene or being hybridizable under stringent conditions to said 5' flanking region.

[0011] The term "isolated DNA sequence" refers to a DNA molecule that is separated from its natural context which means from its naturally occurring chromosome of the organism from which it is derived. For example, the isolated DNA sequence of the invention may be linked to an heterologous coding sequence, inserted into a vector or integrated into the genome of an organism.

[0012] What is meant by "desmin gene" is fully described in the state of the art and summarized in the introductive part of the present application. It comprises coding segments that encodes desmin protein and non coding segments present upstream (5' flanking region), downstream (3' flanking region) and between the coding segments (intron). The gene encoding desmin including its 5' flanking region can be isolated from a commercially available genomic library using an appropriate probe (i.e. cDNA encoding desmin or a portion thereof) or by other conventional molecular biology methods (Polymerase Chain reaction, restriction cutting...).

[0013] The term "5' flanking region" defines the region that is found upstream of the transcription initiation site of a gene. The transcription initiation site is the site at which the first nucleotide of the corresponding transcript is incorporated (where RNA synthesis starts). It can be determined by standard methods such as RNase protection assay. In certain cases, several transcription initiation sites can be identified for a given gene. In this case, transcription initiation site defines the major one which allow the production of the majority (in quantity) of the transcript species. The 5' flanking region contains usually the promoter and at least some of the regulatory elements that regulate (induce or repress) expression of the coding segments under specified conditions. It is within the reach of the man skilled in the art to isolate the specified portion of the desmin 5' flanking region by conventional molecular biology techniques from a genomic library, a desmin gene or from a prior art plasmid.

[0014] The term "approximately" means within a margin of commonly acceptable error for the determination of the size of the DNA sequence being made by standard methods (i.e agarose gel electrophoresis...) or within minor variations for the positions given. A variation of $\pm 10\%$ is acceptable according to the present invention. As used hereinafter, the positions within the 5' flanking region of a desmin gene are given relative to the transcription initiation site which represents nt +1.

[0015] The term "hybridizable under stringent conditions" is conventional in the domain of the art. Such conditions can be found in technical laboratory manuals, such as Maniatis et al., (1982, Molecular cloning, A Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY) or its up to date editions. Preferably, the hybridation conditions are according to the ones described by Maniatis (1982 edition). Three washings are then performed at 65°C in the presence of 0,2 SSC et 0.1 % SDS and without formamide, in order to eliminate the non-hybridized fragments. These conditions denote a percentage of identity between the DNA sequence of the invention and the defined portion of the native 5' flanking desmin region of at least 70%, advantageously 80%, preferably 90% and more preferably of 100%.

[0016] For the purpose of the invention, the DNA sequence of the invention comprises one or more regulatory elements capable alone or in combination, directly or by means of other cellular factors, to allow expression of a gene of interest essentially in muscle cells, advantageously, in vascular smooth muscle cells and preferably in arterial smooth muscle cells and more especially in the aorta and the pulmonary artery. Although background expression can be observed in other tissues (i. e. other muscle cells, fibroblast...etc), expression is observed in majority in the precited target cells. Such a specificity can be determined by *in vitro* transfection assay in different cell types using a gene whose product is easily detectable (LacZ, luciferase, α -antitrypsin, factor IX, CFTR...). Another way to proceed is to generate transgenic mice and evaluate the level of transgene transcript or product in the different tissues.

[0017] However, it is also feasible to employ a functional equivalent of the DNA sequence of the invention having a similar function. Such an equivalent may be obtained by mutation (addition, deletion and/or substitution of one or more nt) or truncation of the native desmin 5' flanking sequences. To illustrate this embodiment, one may refer to a DNA sequence deleted of the negative control regions. It is also possible to mutate or delete some of the *cis*-acting sequences to improve specificity towards vascular or arterial cells or, optionally, decrease specificity towards other tissues. In addition, the DNA sequence of the present invention may comprise exogenous sequences preferably at its 5' or 3' or both 5' and 3' extremities. The capability of the functional equivalent to allow expression of a reporter gene in the target cells can be evaluated *in vitro* or *in vivo* as indicated in the example part.

[0018] According to an advantageous embodiment, the isolated DNA sequence of the invention comprises all or part of the 5' flanking region located between nucleotides -5000 to -1000 relative to the transcription initiation site of said desmin gene.

[0019] For the purposes of the present invention, the desmin gene in use in the present invention, may be from any origin, for example from human, canine, avian, bovine, murine (rat, mouse, hamster...), ovine, feline, porcine or simian origin. However, the DNA sequence of the invention preferably derives from a human or a mouse desmin gene. As already mentioned, the 5' flanking region of this two genes is cloned and is available from GenEMBL (under accession number Z18892 for the mouse gene and M63391 for the human gene).

[0020] According to a preferential embodiment, the DNA sequence of the invention comprises all or part of the 5' flanking region located between approximately nucleotides -4006 to -2495 relative to the transcription initiation site of the mouse desmin gene. In this consideration, it preferably comprises all or part of the sequence shown in SEQ ID NO: 1 or a sequence being hybridizable under stringent conditions. An appropriate part is constituted by at least 100 continuous nt of said SEQ ID NO: 1 and, preferably, at least 200 nt. Of course the DNA sequence may include other sequences homologous or heterologous relative to the desmin gene. In this consideration a DNA sequence deriving from the 5' flanking region located between approximately -4006 to +603 of the mouse desmin gene is preferred.

[0021] According to another alternative, the DNA sequence of the invention comprises all or part of the 5' flanking region located between approximately nucleotides -4000 to -2272 relative to the transcription initiation site of the human desmin gene.

[0022] Another object of the present invention is an expression cassette comprising a gene of interest placed under the control of the elements necessary to its expression in an eukaryotic host cell, said elements comprising at least an isolated DNA sequence according to the invention.

[0023] The expression cassette of the invention can include other elements facilitating maintenance and expression of the gene of interest in host cells, especially from myogenic lineage. In particular, such elements can include a promoter which is operatively linked to the isolated DNA sequence of the invention. Such a promoter is preferably a minimal promoter comprising the *cis*-acting sequences allowing RNA polymerase recognition and binding (TATA box) and optionally (if not present in the gene of interest) a transcription initiation site functional in the targeted host cells.

[0024] Such a promoter may be isolated from any gene of eukaryotic or viral origin. A first possibility is to employ a promoter derived from a desmin gene and comprised in the 5' flanking region immediately upstream of the transcription initiation site and optionally 5' untranslated region of said desmin gene. As far as mouse desmin gene is concerned, such minimal promoter is comprised between approximately nucleotide -87 to -1 of the 5' flanking region of the mouse desmin gene, but functional variants (mutated, truncated) can also be used. Another alternative is to use a heterologous promoter. This latter may be chosen among those of the prior art, for example derived from RSV (Rous Sarcoma Virus), SV40 (Simian Virus 40), HSV (Herpes simplex virus)-TK (thymidine kinase) or CMV (cytomegalovirus) promoter regions, or any promoter especially active in the targeted cell type, especially the minimal promoters of SM22, SM α -actin, SM-MHC, telokin ... etc genes..

[0025] In order to stabilize expression, it may be advantageous that the expression cassette comprises at least an exon, preferably a non coding exon, and optionally an intron. These splicing sequences may derive from the desmin gene, from any eukaryotic gene or from a synthetic sequence. The large variety of splicing sequences described in the state of the art are suitable in the context of the invention. One may cite more particularly those isolated from genes encoding α or β globin, apolipoprotein, immunoglobulin, factor IX, factor VIII, CFTR and from pCI vector (Promega). They are preferably inserted after the transcription initiation site and before the coding sequences. According to the preferred embodiment referring to the mouse desmin gene, one indicates that an appropriate untranslated exon portion extends to approximately nucleotide +79. It is also feasible to include a desmin translated portion to improve translation (i.e. the portion extending to approximately nucleotide +603 of the mouse desmin gene) that is then fused in correct reading frame to the coding sequence of the gene of interest.

[0026] According to the invention, an expression cassette using mouse desmin sequence -4006/+603 operatively linked to a gene of interest is preferred.

[0027] Finally, the expression cassette of the invention may also include a transcription termination signal (poly A) placed downstream of the gene of interest (polyA of SV40...).

[0028] The gene of interest in use in the present invention can be of any origin (prokaryote, eukaryote, viral...) and

isolated from a genomic DNA, a cDNA or be of mixed type (minigene). It can encode an antisense RNA, a ribozyme or a polypeptide. This latter can be a mature polypeptide, a precursor (i.e. precursor intended to be secreted and comprising a signal sequence, a precursor intended to be matured by proteolytic cleavage...), a fragment of a protein (truncated protein), a chimeric polypeptide originating from the fusion of diverse sequences or a mutated polypeptide displaying improved and/or modified biological properties. The gene may be isolated from any organism or cell by the conventional techniques of molecular biology (PCR, cloning with appropriate probes, chemical synthesis) and if needed its sequence may be modified by mutagenesis, PCR or any other protocol.

[0029] Among the suitable genes of interest, one may cite those encoding a polypeptide selected from the group consisting of a cytokine, a chemokine, a membrane or nuclear receptor, a ligand, a clotting factor, a clotting inhibitor, the CFTR protein, insulin, dystrophin, a growth factor (FGF for fibroblast growth factor, NGF for nerve growth factor, VEGF for vascular endothelial growth factor), an enzyme, an enzyme inhibitor, an apoptosis inducer, an apoptosis inhibitor, a cytostatic agent, an apolipoprotein, an oxygen radical scavenger, a cell-cycle control polypeptide, an antiproliferative polypeptide, an angiogenesis inhibitor (angiostatin, endostatin, thrombospondin-1...), an immunogen, an antiviral polypeptide, an antibody, an antibody fragment, a toxin, an immunotoxin, a product of a suicide gene, a muscle contraction inhibiting polypeptide and a marker.

[0030] According to a preferred embodiment, the gene of interest encodes a product capable to convert a prodrug to a cytotoxic drug. One may cite more particularly HSV-TK, which transforms ganciclovir to a guanosine analogue which inhibit DNA synthesis in the replicating cells (Caruso and Klatzmann, 1992, Proc. Natl. Acad. Sci. USA. 89, 182-186), ricin, a bacterial toxin, the expression product of yeast genes *FCYI* and/or *FURI* having UPRTase (Uracil Phosphoribosyl Transferase) and CDase (Cytosine Desaminase) activities.... The gene of interest may also code for a polypeptide having a lipolytic activity (i.e. lipoprotein lipase; see Reyner et al., 1995, Nature Genetics 10, 28; Ming, 1994, J. Biol. Chem 269, 11417). It may also code for a cytostatic polypeptide such as the expression product of Rb gene (Chang et al., 1995, Science 267, 518-522) or of eNos gene (Hamon et al., 1994, Circulation 90, 1357-1362). It can also code for a growth factor of endothelial cells, such as an interleukin.

[0031] Another object of the present invention is a vector comprising an expression cassette according to the invention.

[0032] In the context of the present invention, it can be a plasmid, a synthetic or a viral vector. Plasmid denotes an extrachromosomal circular DNA capable of autonomous replication in a given cell. The choice of the plasmids in use here is very large. It is preferably designed for amplification in bacteria and expression in eukaryotic host cell. Such plasmids can be purchased in a variety of manufacturers. Suitable plasmids include but are not limited to those derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagène), pREP4, pCEP4 (Invitrogen), pCI (Promega) and p Poly (Lathe et al., 1987, Gene 57, 193-201). It is also possible to engineer such a plasmid by molecular biology techniques (Maniatis et al., 1989, Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). A plasmid may also comprise a selection gene in order to select or identify the transfected cells (by complementation of a cell auxotrophy, antibiotic resistance), stabilizing elements (i.e. *cer* sequence; Summers et Sherrat, 1984, Cell 36, 1097-1103) or integrative elements (LTR viral sequences). Furthermore, it can be complexed or associated to synthetic vectors (i.e. liposome, cationic lipids, polymers...).

[0033] An expression vector may also be from viral origin and may be derived from a variety of viruses, such as herpes viruses, cytomegaloviruses, AAV (adeno-associated virus), poxviruses (canarypox, fowlpox, vaccinia virus, MVA) and retroviruses. However, a vector which is specially suitable for the present invention is an adenoviral vector.

[0034] For the purpose of the invention, the adenoviral vector of the invention is preferably replication-defective by deletion of at least all or part of the E1 region. The deletion encompasses at least E1a sequences and may extend in the E1b transcription unit. Preferably the E1b sequences overlapping with pIX gene are not deleted. It goes without saying that the adenoviral backbone of the vector in use in present invention may comprise additional modifications (mutation/deletion/insertion) allocated to one or more viral genes. A partial or total deletion of E3 region may be advantageous.

[0035] A vector of the invention may further comprise an origin of replication efficient in arterial cells, especially smooth muscle cells and/or sequences allowing homologous recombination in the host which can direct replacement of a defective gene by the gene of interest in use in the present invention.

[0036] Another object of the present invention is also a viral particle comprising an expression cassette or a vector according to the invention. It may be prepared according to any conventional technique in the field of the art. When an adenoviral vector is considered, one may proceed by cotransfection of suitable adenoviral fragments in a cell line such as 293 line (as described in Graham and Preveet, 1991, Methods in Molecular Biology, Vol 7, Gene Transfer and Expression Protocols; Ed E.J. Murray, The Human Press Inc, Clinton, NJ). It is also possible to reconstitute the vector in *Escherichia coli* by ligation or homologous recombination (as described in WO96/17070) before transfecting the cell line. Furthermore, the virions may be amplified by passage in a permissive cell in order to generate a high titer viral stock that may be used in the preparation of clinical lots. It may be propagated in a complementation cell line, which supplies *in trans* the deleted/mutated viral functions. Line 293, established from human embryonic kidney cells (Gra-

ham et al., 1977, J. Gen. Virol. 36, 59-72) is commonly used to complement the E1 function. Alternatively, it is also possible to use a helper virus supplying *in trans* some of the viral deficiencies. The viral particles may be recovered from the culture supernatant but also from the cells which can be lysed by a series of thawing/freezing cycles. Optionally, the virions may be amplified and purified according to the standard techniques (chromatography, ultracentrifugation for example in cesium chloride gradient....).

[0037] The subject of the present invention is also an eukaryotic host cell that contains an expression cassette or a vector according to the invention or is infected by a viral particle according to the invention. Preferably, such a cell is from mammalian origin and, in particular from human origin. The vector may be inserted into the cellular genome or not (episome). Suitable cells include but are not limited to primary or tumoral cells from any origin (haematopoietic, muscular, lung, tracheal, liver, epithelial, fibroblastic or endothelial origin). Advantageously, an eukaryotic host cell is a muscle cell, preferably a smooth muscle cell and especially a vascular smooth muscle cell. Among these latter, arterial smooth muscle cells are absolute preference and especially from aorta and pulmonary artery.

[0038] The subject of the invention is also a composition comprising an expression cassette, a vector, a viral particle or an eukaryotic cell according to the invention. It may further comprise one or more substances improving gene transfer efficiency or stability. Such substances are well known in the art (see for example Felgner et al., 1987, Proc. West. Pharmacol. Soc. 32, 115-121; Hodgson et Solaiman, 1996, Nature Biotechnology 14, 339-342; Remy et al., 1994, Bioconjugate Chemistry 5, 647-654, WO94/27238, WO95/10534) and include in particular polymers, polypeptides, cationic lipids, liposomes, nuclear proteins and neutral lipids. They may also be used in combination (i.e. cationic and neutral lipids). Such a substance may also facilitates gene delivery for a particular application. Examples of substances susceptible to facilitate transfection in arterial cells include a gel of a complex of poly-lysine and lactose (Midoux et al., 1993, Nucleic Acids Res. 21, 871-878) or poloxamer 407 (Pastore, 1994, Circulation 90, 1-517).

[0039] According to a preferred embodiment, the composition of the invention is a pharmaceutical composition comprising the precited agents and a pharmaceutically acceptable vehicle. It is intended especially for the preventive or curative treatment of genetic diseases (haemophilia, diabete, cystic fibrosis, Duchenne or Becker myopathies, autoimmune diseases...) or acquired disorders (cancers, tumour, cardiovascular diseases, viral diseases such as AIDS or hepatitis B or C...). A preferred application is the prevention and treatment of cardiovascular disorders.

[0040] The composition according to the invention may be manufactured in a conventional manner for local, general or oral administration. Aerosol, intillation, injection as well as infusion and graft techniques may be envisaged. Suitable routes of administration include subcutaneous, intracardiac, intramuscular, intravenous, intraarterial, intraperitoneal, intratumoral, intrapulmonary, intratracheal routes. The administration may take place in a single dose or a dose repeated one or several times after a certain time interval. The appropriate administration route and dosage vary in accordance with various parameters, for example, with the individual, the disorder to be treated or with the gene of interest to be transferred. The viral particles according to the invention may be formulated in the form of doses of between 10^4 and 10^{14} iu (infectious unit), advantageously 10^5 and 10^{13} iu and preferably 10^6 and 10^{12} iu. The titer may be determined by conventional techniques. Doses based on vector may comprise between 0,01 and 100 mg of DNA, advantageously 0,05 and 10 mg and preferably 0,5 and 5 mg. In addition, the agent of the composition may be combined with a vehicle which is acceptable from a pharmaceutical standpoint. The formulation may also include a diluent, an adjuvant or an excipient. It may be presented as a liquid directly injectable or as a dry powder (lyophilized ... etc) that can be reconstituted before use.

[0041] As far as cardiovascular diseases are concerned, the expression cassette, vector or viral particle may be delivered *in vivo* by specific means adapted to this pathology. One may use balloon catheter that will be inflated in the affected region (Feldman and Steg, 1996, Medecine/Science 12, 47-55). It is also feasible to deliver them directly to the arteries following surgical operation. Another possibility is to introduce along the affected artery a grid frame impregnated with the therapeutic agent (Feldman et al., 1995, J. Clin. Invest. 95, 2662-2671). Graft of smooth muscle cells are also possible.

[0042] Another object of the present invention is the use of an expression cassette, a vector, a viral particle or an eukaryotic cell according to the invention to the manufacture of a drug intended for gene transfer into a host cell or organism and preferably for the treatment of human or animal body by gene therapy. A preferred use is the treatment or prevention of a cardiovascular disease, more especially disorders selected among the group consisting of restenosis following balloon angioplasty, isehemia, intimal hyperplasia, arterosclerosis, hypercholesterolemia and smooth muscle cell proliferation.

[0043] The invention also extends to a method of treatment according to which a therapeutically effective amount of an expression cassette, a vector, a viral particle or an eukaryotic cell according to the invention is administered to a patient in need of such a treatment.

[0044] Another object of the present invention is a transgenic animal, especially a transgenic mouse, comprising an expression cassette or a vector according to the invention. Such an animal can be generated by conventional transgenesis methods and may be used as a model to study the potential effect or activity of the gene of interest or the regulation of the DNA sequence of the invention.

[0045] The invention also concerns the use of a DNA sequence comprising all or part of a sequence located between approximately nucleotides -10000 to +600 of a desmin gene to target expression of a gene of interest operatively linked to said DNA sequence in a muscle cell selected among the group consisting of cardiac, skeletal and smooth muscle cell. A sequence comprised within -4006 to +603 of the mouse desmin gene is preferred. The use of a subfragment (i.e. -10000/-1000 or -4006/-977) operatively linked to an appropriate promoter is also possible

[0046] Finally, the invention concerns the use of a DNA sequence comprising all or part of a sequence located between approximately nucleotides -5000 to -2400 of a mouse desmin gene to target expression of a gene of interest operatively linked to said DNA sequence and to a minimal promoter in an arterial smooth muscle cell and more especially in the aorta and the pulmonary artery. A sequence comprised within -4006 to -2495 of the mouse desmin gene is preferred.

[0047] The present invention is illustrated without being limited by the following examples.

Figure 1 illustrates a comparison of the sequence of the murine and human desmin promoters. Highly conserved regions are shaded. Binding sites for transcription factors are boxed. Transcription start sites are indicated by arrows and numbered +1 (second site for the human sequence).

Figure 2 illustrates the sequence of the arterial SMC specific Desmin [-4006/-2495] region. The putative consensus binding site for transcription factors are boxed. Only the perfect consensus is shown for clarity except for the CArG-like sequences that diverge of 1 nucleotide on 10 compared to the CC(AT)₆GG consensus.

EXAMPLES

[0048] The constructions described below are carried out according to the general techniques of genetic engineering and molecular cloning detailed in Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY) or according to the manufacture's recommendations when a commercial kit is used. Regarding the repair of restriction sites, the technique employed consists of filling in the 5' protruding ends using the large fragment of *E. coli* DNA polymerase 1 (Klenow).

MATERIAL AND METHODS

[0049] The position within the desmin gene and the restriction sites positions (first nt in 5' of the restriction sequence) are given relative to the transcription initiation site (+1) of the desmin gene. Plasmid pE608 was constructed by insertion of a fragment EcoRI (-4005)-Sall (+603) upstream to the marker gene nls-lacZ. The nt G located in 5' of the EcoRI site of the pBluescript ks polylinker is similar to the nt G in position -4006 of the desmin gene. The Sall site (+603) of the desmin is ligated to NcoI site of nls-LacZ sequence after filling in by klenow polymerase, leaving the Sall site reconstructed whereas NcoI site is lost. The desmin sequence is conserved up to position +608 and residue Asp in position 176 is in the correct reading frame relative to the Met residue of nls-lacZ expression products.

[0050] Plasmid pEBB608 was obtained by inserting the blunt ended EcoRI (-4005) - Bst EII (-2500) fragment into BamHI digested and blunt ended pE608 (BamHI sites 5' polylinker and in position -87). As a result the fragment -4004/-2495 is fused to nt -86. Plasmid pXE608 was obtained by inserting the blunt ended XbaI (5' polylinker and -3226) in pE608 deleted of fragment EagI (5' polylinker and -243). In the resulting construct, the sequence -4006/-3222 is fused to position -242 of the desmin sequence.

[0051] To generate pE78, pE608 was deleted of fragment BstEII (-2500)-Sall (+603). The deleted fragment is replaced by a PCR fragment obtained with a sense oligonucleotide overlapping sequence -2503/-2480 (containing BstEII site) and a reverse oligonucleotide overlapping sequence +78/+22, including a Sall site at its 5' extremity and eliminating initiator ATG (+81/+83) of the desmin gene. The desmin sequence GCCACCATG is replaced by GCCAGTCGAC-CATG including a Sall site.

Nuclear extracts

[0052] All procedure were performed at 4°C. All buffers contain cocktail of inhibitors (SIGMA). Cell layer was washed twice and scraped in cold PBS. Cell pellet was resuspended in buffer containing 10 mM HEPES pH8, 50 mM NaCl, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine and 0.2 % triton X100 for 15 min. Cells were lysed with 0.2 % final NP40, 1 min and pelleted 3 min at 2800g. Nuclei pellet were resuspended once in buffer containing 10 mM HEPES pH8, 50 mM NaCl, 20 % glycerol, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine. After 3 min centrifugation (2800 g), nuclei pellet was resuspended in an equal volume of the same buffer except that salt concentration was 350 mM NaCl. To account for pellet volume dilution, 5 M NaCl was added to adjust final NaCl concen-

tration to 350 mM. The resuspended pellet in hypertonic buffer was rotated for 30 min before to be centrifugated 30 min (18300 g). Total protein content of the crude nuclear extract supernatant were quantified by the method of Bradford (Biorad assay) and was usually comprised between 3 to 5 mg/ml. Most of the time, crude nuclear extracts were directly used for EMSA. For footprint analysis, crude nuclear extracts were concentrated by ammonium sulfate precipitation before to be dialysed 4h in buffer containing 10 mM HEPES pH8, 60 mM KCl, 20 % glycerol, 0.25 mM EDTA, 0.125 mM EGTA, 1 mM PMSF, 1 mM DTT.

EMSA

Oligonucleotides used in EMSA analysis

SRE 5' GGATGTCCATATTAGGACATCT
mSRE 5'g.....c.....

Elements CArG de la Desmine

CArG Position

CArG1	5'	AGGATCAGTACATATAAGGCATTATTGT	-1390
CArG2	5'	CATCAAGGACCTTTAGAGGAAAAAGGAG	-2572
CArG3	5'	CTAATAAAGCAAATAAAGGCCAATTTTA	-3443
CArG4	5'	GCAAAACACCCATATATGTAAAATAATT	-3694
mCArG4	5'g.....c.....	
CArG5	5'	TTTAGAAAACCTTTATAGTCCAACCCCC	-3910
CArG6	5'	CCTGGAATTCCTTTTATGCAAATGAGC	-4000
mCArG6	5'g.....c.....	

Oligonucleotides de SM22

SM-CArG1	5'	TGTCTTTCCCCAAATATGGAGCCTGTGT	-150
SM-CArG2	5'	GGGTCCTGCCCATAAAGGTTTTCCTCG	-273
SM-TG	5'	AGAAGGTGGCTTGTTCCTTGCAGGTTCC	-399

[0053] Each CArG-like oligonucleotides was radiolabeled and 12 fmoles of ³²p oligonucleotide was incubated with 4 µg of AUI VSMCS crude nuclear extracts. Competition was made by adding 50x molar ratio of cold oligonucleotide of the same time than the radiolabeled probe.

RESULTS

4006 bp of the murine desmin promoter are sufficient to direct cell specific expression of lacZ in cardiac, skeletal and vascular smooth muscle cells during embryogenesis.

[0054] Reporter genes used in transfection experiments were directly used after linearization to generate transgenic mice that were examined for lacZ expression as founders and stable transgenic lines. We first introduced the larger construct extending from -4006 to +608 bp, namely MDes 4kb-nlz (pE608), to determine what tissue specific elements were contained in that sequence. MDes 4kb-nlz showed to contain sufficient cis-regulatory sequences to direct tissue specific expression of desmin in the three types of muscle, i.e. striated cardiac and skeletal muscles and non-striated smooth muscle cells (SMC).

Cardiac expression

[0055] Whole amount X-Gal staining for lacZ expression revealed that transgene expression was first detected at E7.5 in the late primitive streak embryo. The staining was localised in the bilateral precardiac mesoderm so approximately at the time that the mesoderm cells of this region are fated to cardiac lineage. At E8.5 the transgene was expressed in all regions of the S-shaped primitive heart with an equal intensity. The staining marked the outflow tract, the bulbus cordis, the common ventricle, the common atrial chamber and the sinus venosus. Sections of E9.5 embryos showed that the lacZ positive cells were found in the pericardium and the trabeculated myocardium but not in the endocardium. Desmin-LacZ transgene was never expressed in the endocardial cushion that forms the separation of the atrial and ventricular chambers nor in the fibroblastic tissue of the valve leaflets as shown at E17.5. At each stage, the transgene expression reproduced endogenous desmin expression in the heart as demonstrated by co-localization of the LacZ staining and the immunoperoxidase signal decorating the desmin filaments. In conclusion, desmin transgene as the endogenous protein is specifically expressed in the muscle cells of the heart. High level of expression in the heart persisted throughout adult life.

Vascular Smooth muscle expression.

[0056] The MDes-4kb-LacZ transgene was expressed very early in the smooth muscle cells layer immediately adjacent to the endothelium starting at E9.0, so one day after the appearance of the paired aortae. The paired aortae, the umbilical arteries and the vitelline artery all started to show lacZ staining around the same embryonic stage E9.0. In embryos with 20-22 somites one could see the lacZ staining in the aorta in a double gradient pattern. The strongest staining (or higher number of positive blue nuclei) was observed in both the more rostral paired aortae and the more caudal umbilical arteries. By contrast in the trunk region where the right and left aortae have fused in a single larger vessel, very few positive nuclei were seen at this early stage and the large majority of them were located on the ventral side of the vessel. From stage E10.5, the expression of the Mdes-nlz transgene increased in the dorsal aorta so that the aorta was stained all along its length including the dorsal side. At E12.5, the pattern of expression in the dorsal aorta was regular following the anteroposterior axis. X-Gal staining marked the carotid arteries and the basilar artery in the head at E11.0. Aorta and all main arteries were stained at E15.5 and the transgene continued to be expressed in the arteries throughout adult life.

A transitory expression was observed in the aortic arch arteries that followed the timing of their appearance and transformation into various vessels derivatives. In general less nuclei were stained in the aortic arches compared to the wall of the adjacent paired aortae. Staining in the first aortic arches was observed from E9.0 to E9.5. At this stage the staining was more evident in the second aortic arches and was not visible anymore in E11.0-embryos with 35-40 somites. Third aortic arches began to be stained at E9.5 and number of positive nuclei increased until E11.0. At this later stage, the fourth aortic arches displayed much more positive nuclei than the third aortic arches and only the branching point from the aortae to the sixth aortic arches were stained. In all embryos that were observed at stage E11.0 the aortic arches on the right side of the embryo were less stained than on the left side.

Delayed expression of the transgene in the veins.

[0057] Despite the fact that the main veins like the cardinal veins or the umbilical veins are formed at around the same stage than the arteries, we observed a delay in venous SMCs transgene activation ranging from one to 4 days compared to the arterial SMCs transgene initial expression. Transgene expression in the venous system was first detected in the umbilical veins at E10.5 and at slightly more advanced stage in the vitelline veins but was not clearly visible before E11.0 to E11.5 whereas umbilical and vitelline arteries were already strongly stained at E9.5. During all embryonic development the venous vitelline and umbilical vessels displayed a rather sparse pattern of positive nuclei very different from the dense pattern of positive nuclei in the parallel arterial system including the yolk sac vasculature. Internal large veins like the anterior and posterior vena cavae or the hemizygous vein were not stained before stage E13.5 and were clearly visible at E14.5. Expression of the transgene in the SMCs of the external venous vasculature followed the same timing of activation than the internal veins.

Skeletal muscle expression

[0058] Somite activate MDes-nlz expression in the myotomal compartment as soon as E9.0 following a rostro-caudal gradient reflecting the delayed maturation of the most posterior somites. For instance the last five somites in a 31 somites embryo were not stained. Later the staining was found in the dermomyotomal cells including the thoracic dermomyotomal buds that elongate and invade the ventral body as in the more medial cells forming the axial myotome. Staining was also found in the muscle progenitor cells which had migrated from the somite to limb buds as soon as

E10.5. Staining intensified in the differentiating myofibers from E12.5 to later stages in the axial muscles, ventral body wall muscles and cranio-facial muscles. Ocular muscles appeared to be stained from stage E9.5. Presumably all skeletal muscles were stained by the β -gal activity at E15.5.

5 *Nervous system*

[0059] Beginning at E9.5, the transgene was also expressed in the head externally to the lateral walls of the mesencephalic vesicle. This expression pattern extended anteriorly in the telencephalic vesicles at E12.5 but posteriorly the fourth vesicle stayed negative for lacZ expression. However in the neural tube there was a paired region located in the
10 ependymal layer on each side of the central canal which showed transitory expression of β -gal from E9.5 to E12.5 where it was restricted to the caudal part of the neural tube.

Extra-embryonic expression pattern

15 [0060] As early as stage E6.5 and later on, lacZ expressing cells were also found in the surrounding decidual cells that are from maternal origin. Other sites of extra-embryonic expression were mainly the yolk sac vasculature some sparsely strongly positive cell in the amnion which did not form a vascular network.

[0061] These results demonstrate that the MDes-nlz construct including 4006 pb 5' to the transcription initiation site and 608 bp more of the exon 1, bears all the necessary cis-acting elements able to target tissue specific transcription
20 in cardiac, skeletal and vascular smooth muscle cells but the elements necessary for visceral smooth muscle. Also these results show a delayed activation of the desmin transgene in the venous SMCs compared to the arterial SMCs suggesting transcriptional SMC gene regulation as basis for morphological differences observed between the two types of vascular SMCs.

25 **A distal 1512 bp desmin promoter fragment (pEBB608) directs arterial but not venous SMCs expression**

[0062] A pEBB608 construct was derived from the MDes4kb-LacZ (pE608)s construct by deletion of an internal fragment [-2494/-87] covering the skeletal enhancer region and the downstream half part of the second conserved region with the human promoter (located between -3168 and -1960 of the murine gene). The pEBB608 transgene was
30 analyzed in founder embryos at E15.5. Actually one embryo on three showed a clear dorsal aorta and intercostal vessels staining. Deletion of the fragment [-2494/-87] notably resulted in loss of staining in the heart except for a small population of cells at the outer surface compared to a MDes-nlz embryo at the same stage. A faint specific staining was observed in some of the skeletal myofibers despite the absence of the skeletal enhancer. This could be attributed to the presence of an E-box at position -80, that was shown to confer low level myotube-specific transcription activity. The neu-
35 ral tube staining pattern was slightly different in some founder embryos suggesting that the deleted desmin promoter is susceptible to neural tube cell expression without demonstrating a true specificity.

[0063] Noteworthy was the fact that not all the vessels were stained compared to an equivalent staged MDes4kb-nlz (pE608) embryo. Particularly, histological section analysis of the founder embryo showed that only the arterial SMCs of the aorta and the pulmonary artery but not the SMCs of the vena cava. No other vessels were stained by the X-gal
40 reaction suggesting that the region still present in the deleted transgene are only active in the arterial SMCs of the aorta and the pulmonary system. This observation correlates with an independent regulatory program in the arterial and the venous SMCs.

Promoter sequence analysis

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Sequence Comparison of Murine and Human Desmin Promoter

[0064] A DNA sequence comparison analysis was performed between the human and the murine desmin gene in order to identify the potentially important regulatory regions that would be conserved in the both species. The promoter
50 sequences of the murine (-4447/+695) and the human (-4606/+88) desmin gene were compared using a window stringency algorithm (Compare, GCG Wisconsin University) and a parsimony analysis sequence comparison (Bestfit, GCG Wisconsin University). The dot plot analysis of comparison using the Compare algorithm showed four well conserved segments in the 5' flanking regions (Figure 1, where the conserved regions are shaded) The two first regions lie immediately upstream of the initiation site of transcription and display a high level of nucleotide sequence conservation until
55 -1437 upstream for the murine gene and -1542 for the human gene. The proximal promoter sequences include several conserved transcription factor binding sites like the MyoD I site lying -80 bp upstream of the transcription start site or the Sp1 site at -128 bp for the murine gene. Maximal sequence identity (98 %) was found in the region of the previously identified 275 bp long human skeletal muscle enhancer marked by brackets which is divided in two regions, i.e. the myo-

tube enhancer containing two Mt sites and the MEF2 binding site adjacent to the MyoD I site, the myoblast enhancer containing binding sites for Krox20/24 and Spl factors (Li et al., 1993, J. Biol. Chem. 268, 10403-10415). A second large fragment of sequence conservation was found between -3168 to -1960 bp in the murine promoter and -2940 to -1682 in the human promoter. Two regions display higher level of sequence identity, the first extending from -2196 to -1960 in the murine sequence which did not contain conserved consensus binding sites for known transcription factors, the second well conserved extending from -2946 to -2648 in which consensus binding sites for NF1, AP-1 and HNF-5 were conserved between the two species.

Arterial SMC specific sequence analysis

[0065] Transgenic animals generated with a construct using the 1512 bp sequence between -4004 and -2495 (Fig. 2) that is present in the transgene pEBB608 show an arterial SMC specificity. CArG box motifs are known to regulate many muscle specific gene, especially cardiac and skeletal α -actin gene, via interaction with SRF (Serum Transcription Factor) or other regulatory factors like Nkx 2.5 in the case of the cardiac α -actin gene. Of interest is the fact that amongst 6 CArG-like sequences (90 % of sequence consensus to CC(A/T)₆GG) present in the 4006 bp upstream region, 5 are located in the aortic SMCs specific putative region. We noticed that the CArG-2 motif at position -2572 in the murine sequence is not conserved in the human sequence whereas the slightly divergent CArG-I (ACATATAAGG) at position -1390 in the murine sequence is conserved at 90 % in the human -1479 position but with an even more divergent sequence (ACATGTAAGG) that makes it unlikely to bind SRF. All the 4 other CArG-like motifs in the murine sequence from CArG-3 to CArG-6 lie in a 600 bp long region between -4000 and -3400, so outside the conserved region with the human gene. Other perfect consensus for known transcription factors are shown in figure 2. Interestingly some of them were involved in smooth muscle cell gene regulation. Sp1 is known to regulate positively elastin and PDGFB-R gene in VSMCs and negatively the Sm-MHC gene. The Sp1 zinc finger factor is largely expressed in many tissues, however different expression level was noticed in pups versus adult rat VSMCs where the expression levels were reduced in quiescent SMCs but reinduced in proliferating PDGFB-Rpositive, SM-MHC negative SMCs following arterial wall injury.

Electrophoretic mobility shift assay (EMSA) analysis of the murine CArG box sequences

[0066] Given the known involvement of the SRF transcription factor in the arterial SMC-specific gene regulation and the cluster of the CArG motifs in the putative arterial SMC-specific region of the desmin promoter, we decided to search for potential true SRF binding sites amongst the candidate sequences. EMSA analysis was performed on the CArG boxes of the murine desmin gene. The 6 previously mentioned CArG boxes were chosen on the basis of a 90 % conserved nucleotide sequence relatively to the CC(A/T)₆GG consensus and the conjoined presence of at least one doublet CC or GG at the extremity of the motif. Nuclear extracts from the AUI cell line were used for these experiments. AUI clone is derived from primary human aortic SMCs immortalized by a T-antigen SV40 promoter driven expression (Dr. Dandree). Those cell line express high level of endogenous desmin amongst other SMC-specific marker, a characteristic rather rare in the existing SMCs cell lines,

CArG-4 box located at position -3694 bind a SRF-like molecule

[0067] In a first experiment all 6 CArG-boxes were radiolabelled with ³²P isotope and incubated with AUI nuclear extracts. Only CArG-4 and CArG-6 displayed one (C6) or two (C4) slowly migrating complexes of compatible size with a SRF containing complex. Mutations of the 5' CC doublet to CG and the 3' TG to TC in both the mCArG-4 and the mCArG-6 oligonucleotides abolished the binding of the slowly migrating complexes. To further elucidate the nature of the factors binding to the CArG-4 and CArG-6 motifs, known target sequences for SRF were tested in competition EMSA analysis. Competition was made by adding 50X molar ratio of cold oligonucleotide at the same time than the radiolabeled probe. CArG-4 probe displayed at least 2 specific slowly migrating complexes denoted A and B that were abolished by self-competition and not abolished by a mutated C4 oligonucleotide, and 2 faster complexes that were only partly inhibited by self-competition. Formation of the A-complex was competed by the c-fos SRE, or the SM22 CArG-1 and CArG-2 sequences but not by mutated SRE oligonucleotide, nor by the desmin CArG-6 or an unrelated SM22-TG oligonucleotide. The B-complex was only partly competed by CArG-6 and by the SM22 CArG-1 and CArG-2 sequences. Radiolabeled CArG-6 bound one slow-migrating of similar size than the B complex with CArG-4 but more intense and two faster migrating complex. The B complex was specifically competed by itself and by CArG-4 but not by mC6, mC4 or the SM22 CArG boxes, nor by SRE or SM-TG. These results suggested that SRF or an SRF-like molecule is present in the CArG-4 A complex and possibly in the CArG-4 B complex whereas the CArG-6 B complex was devoid of an SRF molecule. Supershift analysis of the desmin CArG-4 and CArG-6 confirmed this hypotheses. Preincubation of 4 μ g of AUI VSMCs crude nuclear extracts with 2 μ l of SRF antibody directed against the C-terminus of the molecule

(Santa Cruz sc-335X) upshifted the CArG-4 A-complex and the SRE complex migrating at the same size. We noticed that intensity of the bands in the upshifted complexes was decreased suggesting reduction of the SRF-DNA binding affinity by the specific antibody. Those complexes were not upshifted by the pre-immune serum as a control. In the case of CArG-4, the SRF antibody supershifted complex appeared as a doublet and the intensity of the B complex was slightly decreased suggesting that it contained a SRF molecule. The B-complex formed with the CArG-6 oligonucleotide was not supershifted by the SRF antibody nor reduced in signal intensity, showing that this CArG-6 complex did not contain SRF. The same experiment of upshift analysis by the SRF antibody was made using 5 µg of C2.7 myotubes dialysed nuclear extracts. The same results than with the AUI VSMCs nuclear extract were obtained showing that the SRF containing A and B complexes and the CArG-6 B-complex were present in both cell types.

These results showed that the CArG-4 is able to bind a SRF-like molecule as well as another factor also bound by the CArG-6. Interestingly the CArG-6 motif overlapped a perfect ATGCAAAT consensus for Oct factor binding site and this sequence was partially conserved in the same place in the CArG-4 box with the sequence ATGTAAAA. The slightly divergent sequence in the CArG-4 box relative to the consensus should also explain the weaker affinity of the factor for the CArG-4 compared to the CArG-6 oligonucleotide. Moreover the same mutation that abolished the formation of the SRF/CArG-4 complex also abolished the formation of the B complex with both the CArG-4 and the CArG-6 oligonucleotides and one of this mutation changed the ATG5' part of the Oct-consensus in ATC.

DISCUSSION

Vascular Smooth muscle gene regulation

[0068] The MDes4kb-LacZ (pE608) transgene displayed specific activity in vascular SMC expression but not in visceral SMCs. A fragment of 1512 bp between -4004 and -2495 when directly fused to a minimal desmin promoter at position -86 is able to drive lacZ expression in aortic and pulmonary arterial SMCs but not in venous SMCs at E15.5 in a founder embryo. All together these results reinforce the idea that different regulatory programs control gene regulation even from one single gene in the different SMC sub-types. Here we show that the MDes-4kb (pE608) transgenic line expressed lacZ in both the venous and the arterial SMCs and the deleted [-2494/-87] transgene expressed lacZ in a subset of arterial SMCs but not in venous SMCs.

The SMC regulatory program is likely to be slightly different between the murine and the human promoter. One first line of evidence is that CArG-4 box which binds SRF in the murine desmin promoter lies outside the conserved region with the human gene.

Vascular smooth muscle ontogenic process highlighted by the desmin-lacZ transgene expression

[0069] The LacZ staining in the aorta appears at E9.0 in a double gradient pattern from each rostral and caudal paired vessel end. The number of positive nuclei decreases sharply in the trunk region where the right and left aortae have fused in a single larger vessel. Those nuclei are mainly located to the ventral side of the aorta. The preferred ventral localization of the positive nuclei in the developing fused aorta of the mouse embryo could be similar to the early congregation of mesoderm cells to the ventral aortic endothelium of the quail embryo and subsequently the initial expression of SM α -actin and IEL2 antigen in the ventrally located SMCs (Hungerford et al., 1996). Finally the MDes4kb-LacZ (pE608) transgene delayed expression in the venous SMCs compared to the arterial SMCs is noticeable considering the reduced number of smooth muscle cells layer in the veins compared to the arteries. Relative hypomorphic venous SMC development could relate to weaker activation of contractile SMC specific gene promoters.

Skeletal muscle

[0070] From Li et al. (1993a, J. Biol. Chem. 268, 10403-10415; 1993b Development 117, 947-959), the regulation of the desmin gene in the embryonic myotomal precursor cells of the skeletal muscle is clearly understood and is due to a bipartite enhancer located between position -973 and -693 in the human sequence composed of a myotube specific enhancer with a MEF-2 binding site and a MyoD binding site and a myoblast specific enhancer binding Krox and Sp1 family members of transcription factor. This enhancer is highly conserved between in all species for which the sequence is known (hamster, rat, mouse).

However, any of the human or the murine transgene containing only 5' flanking sequence were able to drive expression in the adult skeletal muscles that stopped between birth and two weeks depending on the transgenic lines with residual expression in few dispersed nuclei of some muscles in the adult. Only when intronic and coding sequences were added to the human transgene, adult skeletal muscle expression was maintained. This result suggests that there is a shift of skeletal muscle regulatory program between embryonic and adult life, the former being mainly regulated by the skeletal 5'-located skeletal enhancer and the latter requiring an independent, or alternatively in addition to this enhancer,

regulatory elements located wether in introns or exons or both.

Heart expression

5 [0071] Desmin is expressed very early in the cardiomyocytes precursors arising at E7.5 on each side of the anterior ventral mesoderm. Afterwhat, the gene expression is maintained at high level throughout the embryonic period as well as the adult life. MDes4kb-LacZ transgene recapitulated this pattern of expression in one transgenic line, while another transgenic line only displayed right ventricle expression. In contrast to the decrease and arrest of expression of the different transgenes in the skeletal muscles of the adult mice, the transgenes whether it were from human or murine
10 desmin gene origin, when they were expressed in the embryonic heart, the expression was maintained in the adult heart.

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SEQUENCE LISTING

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(1) GENERAL INFORMATION:

(i) APPLICANT:

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(A) NAME: Universite Paris VII
 (B) STREET: 2 place Jussieu
 (C) CITY: Paris cedex 05
 (E) COUNTRY: France
 (F) POSTAL CODE (ZIP): 75251

15

(ii) TITLE OF INVENTION: Vascular specific regulatory elements
 contained in the desmin 5' flanking region.

(iii) NUMBER OF SEQUENCES: 1

20

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 1512 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Mouse

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GGAATTCCTT TTTATGCAAA TGAGCCATTC CCAGCACCCCT AGCCCCACCA ATCCTGTACT
 60

CAGATAGCCC CAACCATCCC TAGAGGTTTT AGAAAACCTT TATAGTCCAA CCCCCAACTA 120

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AATGTGCTAC TTTCTGAATC CTGTCTTCCT CTCCTTCACT ATCTTGCTCA CTGCTGCCT 180

GAGGTCTCCA TCACCCAAT CTGTAAGTCA GTTCCAGCTG TAGACCTTCT TCTGGACTTC 240

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	TTGGACATCC ATGTGCATCC ATGGAATTCT TGTGCATCAT TTGGGGCACA TACATACATA	300
5	CAGGCAAAAC ACCCATATAT GTAAAATAAT TTTTITTTAA GATTAGCACC TTAGTTTACT	360
	TTTTGACAGT TTTTTCCTTG TTTGCTTGGT TAGTTGGTTG GTTTGGCTTT TTGAGATAAC	420
	GTTTCTCTGT GTAGTCTTGG CTGTCCTGAA ATTCACACTA TGTAGACCAG GCTAGCTTTG	480
10	AACCCACAGA GATCTGGCTG CCTCTGCTGG GATTAAAGCA TAGGCCACCA CGATTGGCTT	540
	TGAGAAGAAA TAAACTAATA AAGCAAAATA AGGCCAATTT TATGTCACTC TATTTTATAA	600
	CGATAAACAT GACTTAGTTA TGAGGGAAGT GAGACAGGAC ACTTCTAAGA CACCTGTGTT	660
15	TCTGACAACA GCCTTACCTC AGAGCAGCAT GTAAAATATT TTAATAAGGG AAAAAGCCAG	720
	GTGTGGTAAT GCTCACCTGT AATCCTAGCC CTCTGGAAGT TAAGGCAGGA AAATCATGAG	780
20	TCTAGACTAC AAAAAGAAAA AATAAAGGTC AGCTCCACAT ACGAGATCAA CACCTCCAC	840
	CACCTAAACT TGGTCCTATG GCTCAGGGGC TGGGGCGGGC TGAATAGTT AACTGCTGAA	900
	AAAACGGGAT CCAGTAACTT TTCCCTTTAA GCTCTTTTTT TATACCCTGT GTTTTGATCG	960
25	TGAGCATTGC CCATATGGGA AGATAGTGAT TATTTTAGCG TCCAGTCCC AACGGCCCCC	1020
	TTGGAAGCC AAGGTCTTGC TCCAGCGCCT TCTCAGAGTG TGTFTGGGG TGTTCCTTTT	1080
	TCTCCTTCCT GCCTTCAGG CCCCAGAGTG TTGTTTACT GCTCGCCCTG GCTGTGTCCT	1140
30	CAGGAGTGA ACCACATCTT GGAAAAAGTC CCAGTTGGTT TGAAGCCAAG AGCCTGGTAG	1200
	TTGTTGATTT TGAAGTAGA TGATGCGGTT TATTTATTTG TTTCTGCCT CATTCACCA	1260
35	AGCCAACTGA AGGCTAGGGC GTGAAGGCCA TTCCCTTATG TGTCTAGGG ATCCTCGCAA	1320
	AGCCCCAGCA ATGTCCTTCT TAGCAACTTC ATAGAAATGT CCTATACCTT CACAGCAATG	1380
	TCCTTCTACA CCCTGTTTCT TTGGATTGCA GATGACTAGA GCCAACATCA AGGACCTTTA	1440
40	GAGGAAAAAG GAGTTTCATG ATCCAAGTAG GGGACAAAGC CTGACAAGGG CCTAGGAGTT	1500
	CGAGCTGGTG AC	1512
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Claims

1. An isolated DNA sequence comprising all or part of the 5' flanking region of a desmin gene located between approximately nucleotides -10000 to -1000 relative to the transcription initiation site of said desmin gene or being hybridizable under stringent conditions to said 5' flanking region.
2. An isolated DNA sequence according to claim 1, wherein said 5' flanking region is located between nucleotides -5000 to -1000 relative to the transcription initiation site of said desmin gene.
3. An isolated DNA sequence according to claim 1 or 2, wherein said desmin gene is from human, canine, avian, bovine, murine, ovine, feline, porcine or simian origin.

4. An isolated DNA sequence according to claim 3, wherein said 5' flanking region is located between approximately nucleotides -4006 to -2495 relative to the transcription initiation site of the mouse desmin gene.
5. An isolated DNA sequence according claim 4, wherein said DNA sequence comprises all or part of the sequence shown in SEQ ID NO: 1 or a sequence being hybridizable under stringent conditions.
6. An isolated DNA sequence according to claim 3, wherein said 5' flanking region is located between approximately nucleotides -4006 to +603 relative to the transcription initiation site of the mouse desmin gene.
7. An expression cassette comprising a gene of interest placed under the control of the elements necessary to its expression in an eukaryotic host cell, said elements comprising at least an isolated DNA sequence according to claims 1 to 6.
8. An expression cassette according to claim 7, wherein said elements further comprise a promoter, preferably a minimal promoter, operatively linked to said isolated DNA sequence.
9. An expression cassette according to claim 8, wherein said minimal promoter is derived from a desmin gene and comprised in the 5' flanking region immediately upstream of the transcription initiation site of said desmin gene.
10. An expression cassette according to claim 9, wherein said minimal promoter is comprised between approximately nucleotide -87 to -1 of the 5' flanking region of the mouse desmin gene.
11. An expression cassette according to any of claims 7 to 10, wherein said elements further comprise at least an exon, preferably a non coding exon, and optionally an intron.
12. An expression cassette according to claim 11, wherein said exon is non coding and extends to approximately nucleotide +79 of the mouse desmin gene or comprises coding sequence and extends to approximately nucleotide +603 of the mouse desmin gene.
13. An expression cassette according to any of claims 7 to 12, wherein said gene of interest encodes an antisense RNA, a ribozyme or a polypeptide.
14. An expression cassette according to claim 13, wherein said polypeptide is selected from the group consisting of a cytokine, a chemokine, a membrane or nuclear receptor, a ligand, a clotting factor, a clotting inhibitor, the CFTR protein, insulin, dystrophin, a growth factor, an enzyme, an enzyme inhibitor, an apoptosis inducer, an apoptosis inhibitor, a cytostatic agent, an apolipoprotein, an oxygen radical scavenger, a cell-cycle control polypeptide, an anti-proliferative polypeptide, an angiogenesis inhibitor, an immunogen, a antiviral polypeptide, an antibody, an antibody fragment, a toxin, an immunotoxin, a product of a suicide gene, a muscle contraction inhibiting polypeptide and a marker.
15. A vector comprising an expression cassette according to any of claims 7 to 14.
16. A vector according to claim 15, wherein said vector is selected among plasmid, synthetic and viral vectors.
17. A vector according to claim 16, wherein said vector is an adenoviral vector.
18. A vector according to claim 17, wherein said adenoviral vector is replication-defective by deletion of at least all or part of the E1 region.
19. A viral particle comprising an expression cassette according to any of claims 7 to 14 or a vector according to any of claims 15 to 18.
20. An eukaryotic host cell, wherein said cell contains an expression cassette according to any of claims 7 to 14 or a vector according to any of claims 15 to 18 or is infected by a viral particle according to claim 19.
21. An eukaryotic host cell according to claim 20, wherein said cell is a muscle cell, preferably a smooth muscle cell.
22. An eukaryotic host cell according to claim 21, wherein said cell is a vascular smooth muscle cell.

23. An eukaryotic host cell according to claim 22, wherein said cell is a arterial smooth muscle cell.
24. A composition comprising an expression cassette according to any of claims 7 to 14, a vector according to any of claims 15 to 18, a viral particle according to claim 19 or an eukaryotic cell according to any of claims 20 to 23.
25. A composition according to claim 24, further comprising a substance facilitating transfection or stability, such as a gel of a complex of poly-lysine and lactose or polyoxamer 407.
26. A pharmaceutical composition comprising an expression cassette according to any of claims 7 to 14, a vector according to any of claims 15 to 18, a viral particle according to claim 19 or an eukaryotic cell according to any of claims 20 to 23 and a pharmaceutically acceptable vehicle.
27. Use of an expression cassette according to any of claims 7 to 14, a vector according to any of claims 15 to 18, a viral particle according to claim 19 or an eukaryotic cell according to any of claims 20 to 23 to the manufacture of a drug intended for the treatment or prevention of a cardiovascular disease.
28. Use according to claim 27, wherein the cardiovascular disease is selected among the group consisting of restenosis, ischemia, intimal hyperplasia, arteriosclerosis, hypercholesterolemia and smooth muscle cell proliferation.
29. Transgenic animal comprising an expression cassette according to any of claims 7 to 14 or a vector according to any of claims 15 to 18.
30. Use of a DNA sequence comprising all or part of a sequence located between approximately nucleotides -10000 to +600 of a desmin gene and preferable -4006 to +603 of the mouse desmin gene, to target expression of a gene of interest operatively linked to said DNA sequence in a muscle cell selected among the group consisting of cardiac, skeletal and smooth muscle cell.
31. Use of a DNA sequence comprising all or part of a sequence located between approximately nucleotides -5000 to -2400 of a desmin gene and preferably -4006 to -2495 of the mouse desmin gene, to target expression of a gene of interest operatively linked to said DNA sequence and to a minimal promoter in an arterial smooth muscle cell.

- Figure 1 -

Mouse -3168 ACCACCTAACTTGGT...CTATGGCTCAGGGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -3073
 Human -2935 ACAACCAAACTCTGGTGTCTCTGAAGCAGTGTGTTGGGGTGGCTGG...TGTCAAAGGGGGAAGGAGACTTACTTTTCATTTATGC.C -2850
 Mouse -3072 TTTTATTATACCTGTGTTTG.ATCGTGAGCATTGCCATATGGGAAGATAGTGATTATTT...AGCGTCAGTTCACCGGCCCCCTTGGAAAGCC -2977
 Human -2849 TTTTGCACATCTTGAATTTGTACCATATACACTACCCATCCAAATCCCTGTGGTATTTTATGTGTGCC.CATCCGAGTGGCTCTCTCGG.AGGET -2752
 Mouse -2976 AAGGTCTTGTCTCCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2884
 Human -2751 GAACTCTCCCTCCAGCGGTGCTGCCAGCTCAGCACTGTTTGGGGTGGCTGG...TGTCAAAGGGGGAAGGAGACTTACTTTTCATTTATGC.C -2658
 Mouse -2883 CTTCTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2786
 Human -2657 CTTCTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2563
 Mouse -2785 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2692
 Human -2562 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2465
 Mouse -2691 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2605
 Human -2464 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2379
 Mouse -2604 GGATTGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2509
 Human -2378 GGGCTGTAAATGATCAAG.CAATACCAATGCTTAAATTAATAAAAAAAAAAAAAAAAAAAGGAAAGTTTGTGCCAAGGCCAGGAAGAC...AGTG -2283
 Mouse -2508 TTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -2430
 Human -2282 TGGGAGC...TCAACTCACAAGTTGAGGGAAGCACTGCAAGAACCAAGGGGCTGGCTGCTCTCTCAGCCTCTCTGCTT...CTTACATATT -2190
 Mouse -2429 GACCCAC...CCCACCCCAACAGAGTCAGACA...GGGACCAAGG.ACCAGTTTACCAAAACCCCAATTTATTCATAAATTCAG...AGATC -2342
 Human -2189 GACCTCTCTTTCTTCTACTCTCCAGGGGGCAGGAACATGGCTTCCAGAGTTCAGTTGAAGATCCAGTTCCGTCTATAAATTCAGGGAAGGTC -2090
 Mouse -2341 TCAGCTTGACCTGCTCAGAAC...CACACCTTGAGCAATTTTACAGTCAAGGAATGC...AATGGCAGGATCTGAGTCATGTATCT -2254
 Human -2089 TCTGATGGCCCTACTCATTCCAGGCCATTCTTGACCAAGTCACTGAAGTCAGGAGATGCAATAAGACTGGCTGGAATCAGGGTC...TTT -1995
 Mouse -2253 ACTCGTTGAG...CTTCAAGAGTGAAGTGTGTCTCTCAGAGAACCAAGGCCAGAA...AATGGCAGGATCTGAGTCATGTATCT -2176
 Human -1994 AGGGGTGGAGGATGGGAGGAGGACAGCATGTATCAATAAGCAATTGCAAAAGAAAGCTTGCAGGCTACTTTGATGCAATCAGAA.AGACCG -1896
 Mouse -2175 TGTGGGCTGGGATGGGAGGAGGACAGCATGTATCAATAAGCAATTGCAAAAGAAAGCTTGCAGGCTACTTTGATGCAATCAGAA.AGACCG -2076
 Human -1895 TGTGGGCTGGGATGGGAGGAGGACAGCATGTATCAATAAGCAATTGCAAAAGAAAGCTTGCAGGCTACTTTGATGCAATCAGAA.AGACCG -1798
 Mouse -2075 AGGCTTGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -1980
 Human -1797 AGGCTTGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -1698
 Mouse -1979 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -1960522 bp.....
 Human -1697 GATTCGAGTGTGCTCAGG...CCTTCTCAGAGTGTGTTGGGGTGGCTGGGGGGGCTGGAATAGTTAACTGCTGAAAAACGGGATCCAG...TAAGTTTCCCTTTAAGCTC -1677134 bp.....

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- Figure 2 -

-4006 ^{CAR6} ^{Oct} ^{EcoR I} GGAATTCCTT TTTATCAAA T ^{GAGCCATTC} ^{Sp1} CCAGCAGCCT AGCCCCACCA
 -3956 ATCTGTACT ^{Gata} CAGATAGCCC CAACCATCCC TAGAGGTTTT AGAAA ^{CAR5} CCTT
 -3906 TATAGTCAA CCCCCAATA AATGTGCTAC TTTCTGAATC CTGTCTTCT
 -3856 ^{Gata} CTCCTCACT ATCTTGCTCA CTGCTGCT GAGGTCTCCA TCACCCCAAT
 -3806 CTGTAAGTCA GTCCAGCTG TAGACCTTCT TCTGGACTTC TTGGACATCC
 -3756 ATGTGCATCC ATGGACTTCT TGTGCATCAT TTGGGGCACA TACATACATA
 -3706 CAGGCAAAAC ^{NKX2.5} ^{CAR4} ACCATATAT GTAAAATAAT TTTTTTTAA GATTAGCACC
 -3656 TTAGTTTACT TTTTGACAGT TTTTGCTTG TTTGCTTGGT TAGTTGGTTG
 -3606 ^{Gata} GTTTGGCTTT TTGAGATAAC GTTTCTCTGT GTAGCTCTGG CTGTCTGAA
 -3556 ATTCACACTA TGTAGACCAG GCTAGCTTTG AACCCACAGA GATCTGGCTG
 -3506 CCTCTGCTGG GATTAAAGCA TAGGCCACCA ^{NF-1} CATTGGCTT TGAGAAGAAA
 -3456 TAAACTAATA AAGCAAATAA AGGCCAATTT ^{NKX2.5} TATGTCATCTC TATTTTATAA
 -3406 CGATAAACAT GACTTAGTTA TGAGGGAAC T GAGACAGGAC ACTTCTAAGA
 -3356 CACCTGTGTT TCTGACAACA ^{C/EBP} GCTTACCTC AGAGCAGCAT GTAAAATATT
 -3306 ^{YY1} TTAATAAGGG AAAAAGCCAG ^{C/EBP} GTGTGTAAT GCTCACCTGT AATCCTAGCC
 -3256 CTCTGGAAGT TAAGGCAGGA AAATCATGAG TCTAGACTAC AAAAAGAAAA
 -3206 AATAAAGGTC AGCTCCACAT ACGAGATCAA CAACCTCCAC CACCTAACT
 -3156 TGGTCTATG GCTCAGGGGC ^{Sp1} ^{Sp1} TGGGCGGGC TGGAAATAGTT AACTGCTGAA
 -3106 AAAACGGGAT CCAGTAACTT TCCCTTTAA GCTCTTTTTT TATACCCTGT
 -3056 GTTTTGATCG TGAGCATTGC CCATATGGGA ^{Gata} AGATAGTGAT TATTTAGCG
 -3006 TCCAGTCCC AACGGCCCCC TTGGAAGCC AAGGTCTTGC TCCAGCGCT
 -2956 TCTCAGAGTG TGTTTGGGG TGTTCTTTT TCTCCTTCTT GCCTTTCAGG
 -2906 CCCCAGAGTG TTGTTTACT GCTGGCCCTG ^{AP-2} GCTGTGCTT CAGGAGTGA
 -2856 ACCACATCTT GGAAAAAGTC CCAGTGGGT ^{NF-1} TGAAGCCAAG AGCCTGGTAG
 -2806 TTGTTGATTT ^{AP-1} TGACTAGAGA TGATGCGGT TATTTATTG TTTCTGCCT
 -2756 CATTTCACCA AGCCAAGTGA AGGCTAGGGC GTGAAGGCCA TCCCTTATG
 -2706 TGTTCTAGGG ATGCTCGCA AGCCCCAGCA ^{YY1} ATGCTTCT TAGCAACTTC
 -2656 ATAGAAATGT CCTATACCTT CACAGCAATG TCCTTCTACA CCCTGTTTCT
 -2606 TTGGATTGCA ^{AP-1} GATGACTAGA GCCAACATCA ^{CAR2} AGGACCTTA GAGGAAAAAG
 -2556 GAGTTTCATG ATCCAAGTAG GGGACAAAGC CTGACAAGGG CCTAGGAGTT
 -2506 CGAGCTCTGAC -2495
 Bst EII b1nt



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INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>Although claims 30 and 31 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.</p>			
Place of search		Date of completion of the search	Examiner
THE HAGUE		24 March 1999	Oderwald, H
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

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